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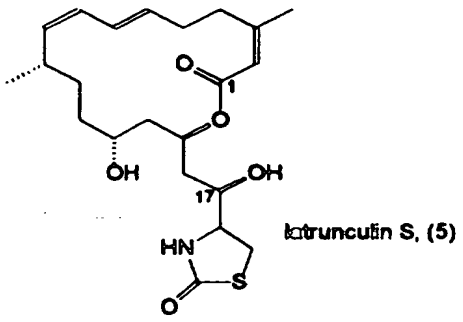
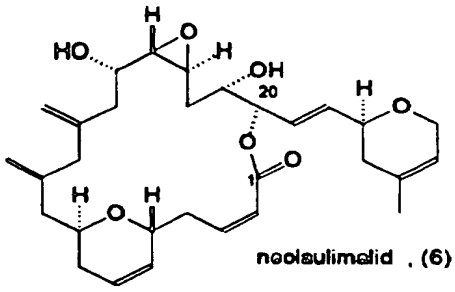
(54) Title: THREE NEW CYTOTOXIC MACROLIDES FROM A MARINE SPONGE

(57) Abstract

Novel antitumor compounds latrunculin S, neolaulimalide and zampanolide, also respectively referred to as compounds (5, 6, and 7), of formulae (5, 6 and 7), can be isolated from the sponge $(i(Fasciospongia rimosa).



latrunculin S, (5)



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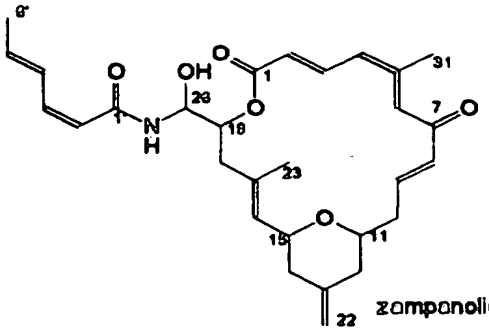

zamponolide, (7)

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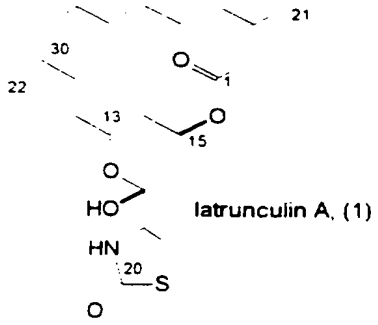
Three New Cytotoxic Macrolides from a Marine Sponge

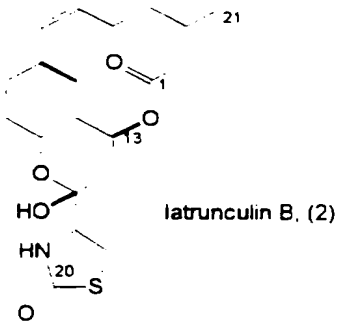
The present invention relates to new macrolides from marine sponge. Such compounds have antitumor activity.

BACKGROUND OF THE INVENTION

Ichthyotoxic sponge metabolites designated the latrunculins were first reported by Kashman et al., see Tetrahedron Lett., 1980, 21, 3629; and J. Org. Chem., 1983, 49, 3512, as novel marine macrolides having significant biological activity. Apart from potent toxicity against fish, latrunculin A (compound 1) and latrunculin B (compound 2) have been shown to effect strong reversible alteration of the microfilament organisation in cultured cells, see Science 1983, 219, 493.



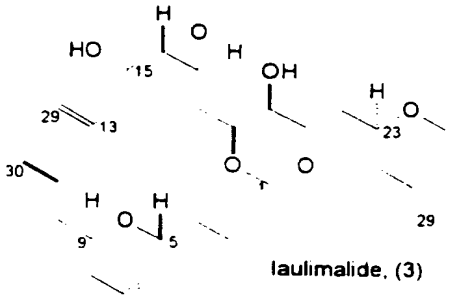
latrunculin A, (1)



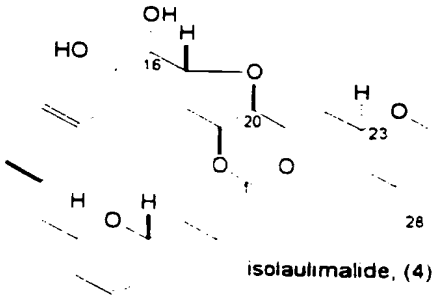
latrunculin B, (2)

Latrunculins have since then been isolated from other sponge species and nudibranchs. Most notably, latrunculin A and the laulimalides (or

fijianolides, compounds 3 and 4), another class of macrolides, have been reported to co-occur in three specimens of Pacific sponges described as *Hyatella* sp., see J. Org. Chem., 1988, 53, 3644, *Spongia mycofijiensis*, see J. Org. Chem., 1988, 53, 3642, and unidentified species, see J. Nat. Prod., 1992, 55, 506.



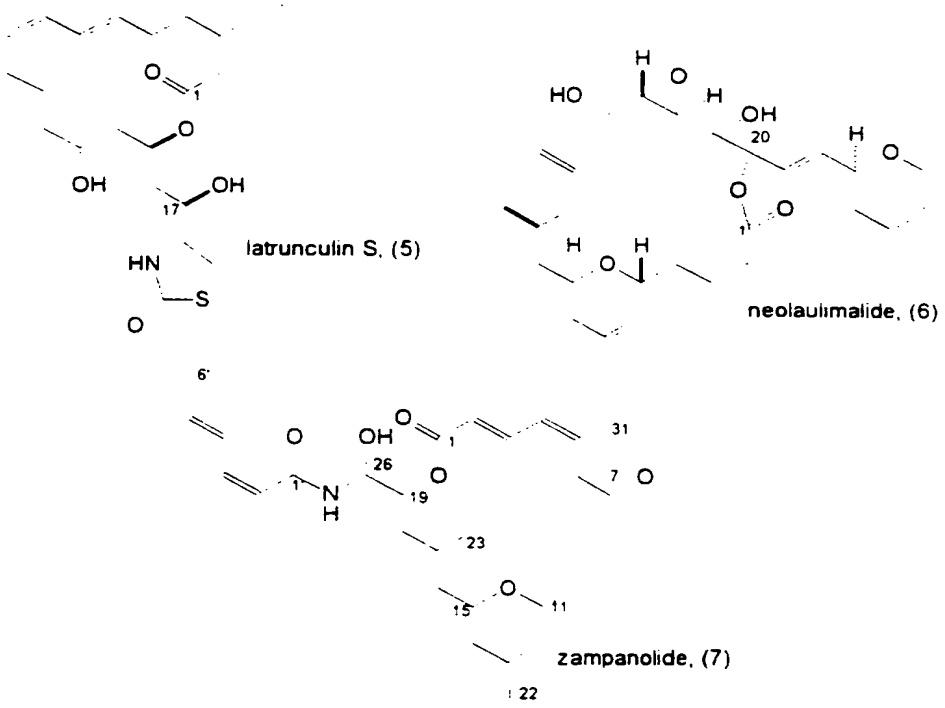
laulimalide, (3)



isolaulimalide, (4)

SUMMARY OF THE PRESENT INVENTION

The present invention provides compounds designated latrunculin S, neolaulimalide and zampanolide, also referred to as compounds (5), (6) and (7). The structures of these three compounds are as follows:



The compounds show antitumor activity. Thus, the invention also provides antitumor compositions and methods using at least one of the compounds of this invention.

A method of isolating the compounds is also provided, by extraction from the sponge *Fasciospongia rimosa.*

EMBODIMENTS OF THE INVENTION

Examples of pharmaceutical compositions provided by this invention include solid (tablets, pills, capsules, granules, etc.) or liquid (solutions, suspensions or emulsions) formulations with a suitable composition for oral, topical or parenteral administration. They may contain the pure compound or

in combination with any other pharmacologically active compound. These compositions may need to be sterile when administered parentally.

The correct dosage of pharmaceutical composition comprising a compound of the invention will vary according to the pharmaceutical formulation, the mode of application, and the particular situs, host and tumor being treated. Other factors like age, body weight, sex diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction sensitivities and severity of the disease shall be taken into account. Administration can be carried out continuously or periodically within the maximum tolerated dose.

Compounds of the invention can be made by isolation from marine sources, notably by a process of this invention which comprises extraction from *Fasciospongia rimosa*, or by synthetic or semi-synthetic procedures.

EXAMPLES OF THE INVENTION

The present invention is illustrated by the following Examples, which include details of the isolation of the compounds from marine sponge *Fasciospongia rimosa* collected in Okinawa and for which a voucher specimen (G301467) was deposited at Queensland Museum, Australia, and details of the biological activity of the compounds. A second voucher specimen (QMG312707) has been deposited at the same Museum.

Taxonomically, the correct generic and family assignments of the sponge are a problem, which possibly is more correctly identified as "*Hyattella*" *rimosa* (Lamarck) (order dictyoceratida: family ?Spongiidae). P Bergquist noted in the reprint by Gulavita, Gunasekera and Pomponi (1992) (J Nat. Prod. 55(4): 508), this "species" strictly belongs to the family Thorectidae because it has lamellated fibres. It has previously been called *Spongia mycofijiensis Bakus* (e.g. Quinoa, Kakou & Crews (1998), J. Org. Chem, 53:3644), a "new genus" of Thorectidae (Gulavita, Gunasekera and Pomponi (1992) J. Nat. Prod. 55($):508), and *Fasciospongia rimosa* (Lamarck) in the present work..

Isolation

The sponge *Fasciospongia Rimosa* was collected from underwater caves on Shimoji-jima, an island located in the southwest of Okinawa. A sample (wet. 4.48 kg) was extracted by steeping in acetone, and the residue after concentration was reextracted with EtOAc to give 39 g of an oil. The oil showed potent cytotoxicity (IC_{50} 0.002-0.1 μg/ml) against P388, A549, and HT29 cell lines. Separation of the extract as shown in Scheme 1 gave latrunculin A (compound 1, 17.2% of the extract), laulimalide (compound 3, 4.2%), isolaulimalide (compound 4, 0.31%), and two new minor constituents designated as latrunculin S (compound 5, 0.012%) and neolaulimalide (compound 6, 0.012%). We originally named latrunculin S as latrunculin E, but a compound named latrunculin E already exists in the literature.

A second collection (480 g) of the sponge from the island of Okinawa was similarly treated to furnish latrunculin A (compound 1, 35% of EtOAc extract) and a new macrolide named zampanolide (compound 7, 0.13%). We originally named zampanolide as fasciolide, but prefer zampanolide to reflect the name of collection site of the sponge.

Structures

Table 1 shows the molecular formulae and some physical properties for the new compounds 5, 6 and 7. Comparison of the ^{1}H and ^{13}C NMR spectra (Figure 2) with those of latrunculin A (compound 1) and 2D NMR analysis suggested the structure of latrunculin S to be depicted as shown for compound 5. Structural correlation of compound 5 with compound 1 was secured by $NaBH_4$ reduction of compound 1 which yielded two diastereomeric products compound 5 and compound 8 (Scheme 2). One of them was identical with latrunculin S (compound 5). The absolute configuration at C17 or 5 was *R*, as determined by modified Mosher's method.

Table 1. Molecular Formula and Physical Data for Compounds 5-7

Compound	Latrunculin S (5)	Neolaulimalide (6)	Zampanolide (7)
HRMS (m/z)	423.2083 $(M)^+$	515.3008 $(M^+ + 1)$	496.2683 $(M^+ + 1)$
MF	$C_{22}H_{33}NO_5S$	$C_{30}H_{42}O_7$	$C_{29}H_{37}NO_6$
$[\alpha]_D$	+110°	-57°	-101°
IR	3540.1694	3620.1715	1680.1645

Neolaulimalide (6) had the same molecular formula $C_{30}H_{42}O_7$ with those of laulimalide (3) and isolaulimalide (4), suggesting a related isomeric structure. The structure (6) was determined by 2D NMR analysis including COSY, TOCSY, HMQC and HMBC. The assignment of NMR data is shown in Figure 3. The structure and absolute stereochemistry of compound 6 were confirmed by conversion to isolaulimalide (4). Treatment 6 with CSA gave 4 as a major product. Progress of the conversion reaction could be monitored by NMR as shown in Figure 4. Although the conversion of laulimalide (3) to isolaulimalide (4) in the same treatment was complete in 2 hr, the conversion of 6 took more than 48 hr to complete (Scheme 3).

The molecular formula of zampanolide (7) was found to be $C_{29}H_{37}NO_6$ by HRFABMS. The structure was elucidated by analysis of 2D NMR spectra (BCOSY, TOCSY, HMQC, HMBC and PSNOESY, Figures 5-6) as a new 20-membered macrolide having an amide of a 2,4-hexadienoic acid on the side chain.

Further physical data for these compounds is given in Chemistry Letters 1996 pp 255,256 and Tetrahedron Letters Vol. 37 No. 31, pp 5535-5538, 1996

Biological Activity.

Cells were maintained in logarithmic phase of growth in Eagle's Minimum Essential Medium, with Earle's Balanced Salts, with 2.0 mM L-glutamine, with non-essential amino acids, without sodium bicarbonate (EMEM/neaa); supplemented with 10% Fetal Calf Serum (FCS), 10^{-2} M sodium bicarbonate and 0,1 g/l penicillin-G + streptomycin sulphate.

A simple screening procedure was carried out to determine and compare the antitumor activity of these compounds, using an adapted form of a literature method. The antitumor cells employed were P-388 (suspension culture of a lymphoid neoplasm from DBA/2 mouse), A-549 (monolayer culture of a human lung carcinoma), HT-29 (monolayer culture of a human colon carcinoma) and MEL-28 (monolayer culture of a human melanoma).

P-388 were seeded into 16 mm wells at 1 x 10^4 cells per well in 1 ml aliquots of MEM 5FCS containing the indicated concentration of drug. A separate set of cultures without drug were seeded as control growth to ensure that these cells remained in exponential phase of growth. All determinations were carried out in duplicate. After three days of incubation at 37°C, 10% CO_2 in an atmosphere of 98% humidity, the wells were stained with 0.1% Crystal Violet. An approximate IC_{50} was determined by comparing the growth in wells with drug to the growth in wells control.

A-549, HT-29 and MEL-28 cells were seeded into 16 mm wells at 2 x 10^4 cells per well in 1 ml aliquots of MEM 10FCS containing the indicated concentration of drug. A separate set of cultures without drug was seeded as control growth to ensure that cells remained in exponential phase of growth. All determinations were carried out in duplicate. After three days of incubation at 37°C, 10% CO_2 in an atmosphere of 98% humidity, the wells were stained with 0.1% Crystal Violet. An approximate IC_{50} was determined by comparing the growth in wells with drug to the growth in wells control.

Results:

	IC_{50} µg/ml			
Compound	P-388	A-549	HT-29	MEL 28
latrunculin S	1.2	0.5	1	1
neolaulimalide	0.05	0.01	0.025	0.025
zampanolide	0.001	0.005	0.005	0.005

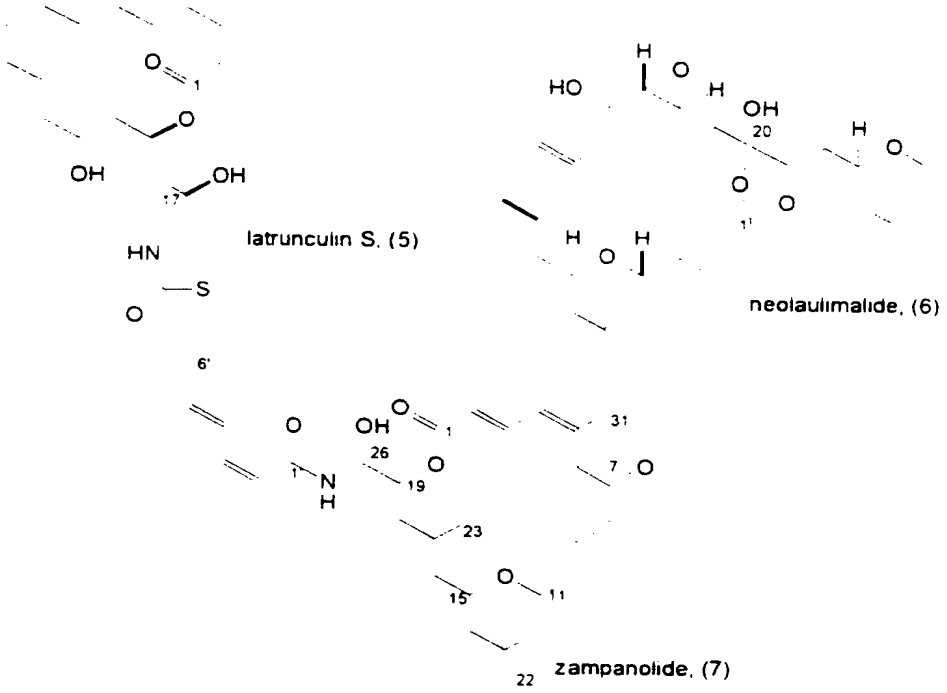
Literature References

Biochem. Bioph. Res. Comm. 1984. 3. 848-854.

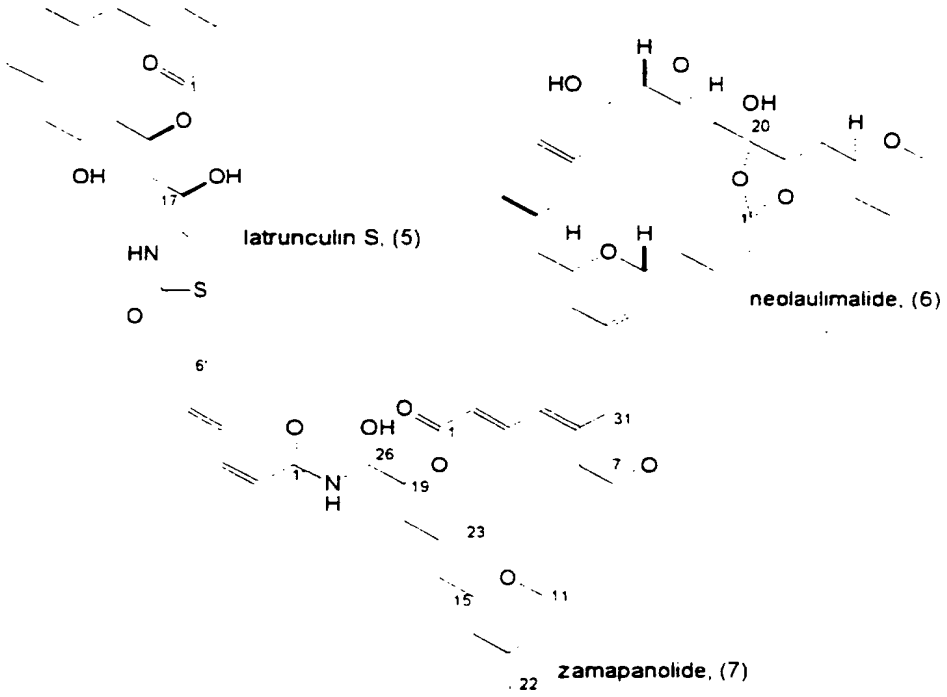
J. Med. Chem 1981. 24. 1078-1083.

CLAIMS

1. A compound selected from the group consisting of latrunculin S, neolaulimalide and zampanolide, also respectively referred to as compounds (5), (6) and (7), of the following formulae:

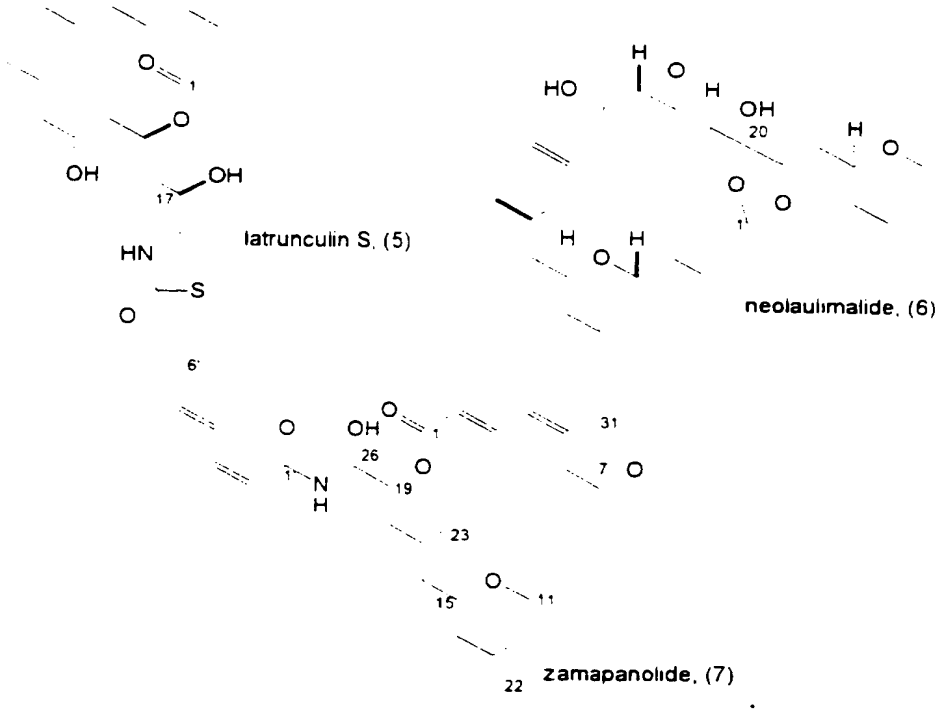


2. A pharmaceutical composition containing a compound selected from the group consisting of latrunculin S, neolaulimalide and zampanolide, also respectively referred to as compounds (5), (6) and (7), of the following formulae:

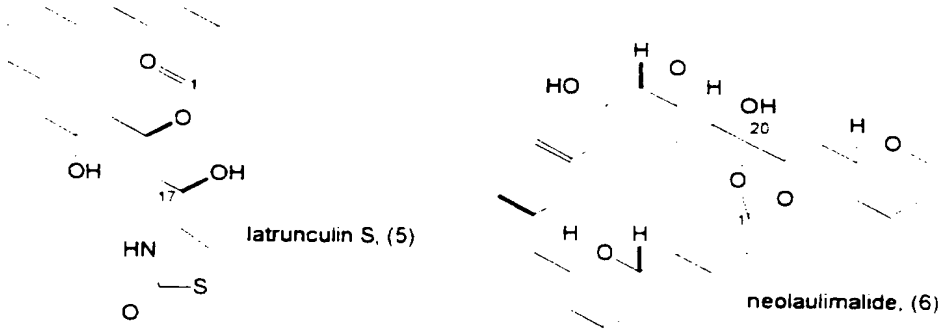


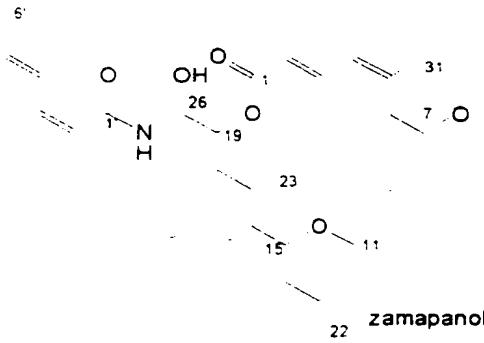
together with a pharmaceutically acceptable carrier.

3. A method for treatment of a tumor which comprises administering an effective amount of a compound selected from the group consisting of latrunculin S, neolaulimalide and zampanolide, also respectively referred to as compounds (5), (6) and (7), of the following formulae:



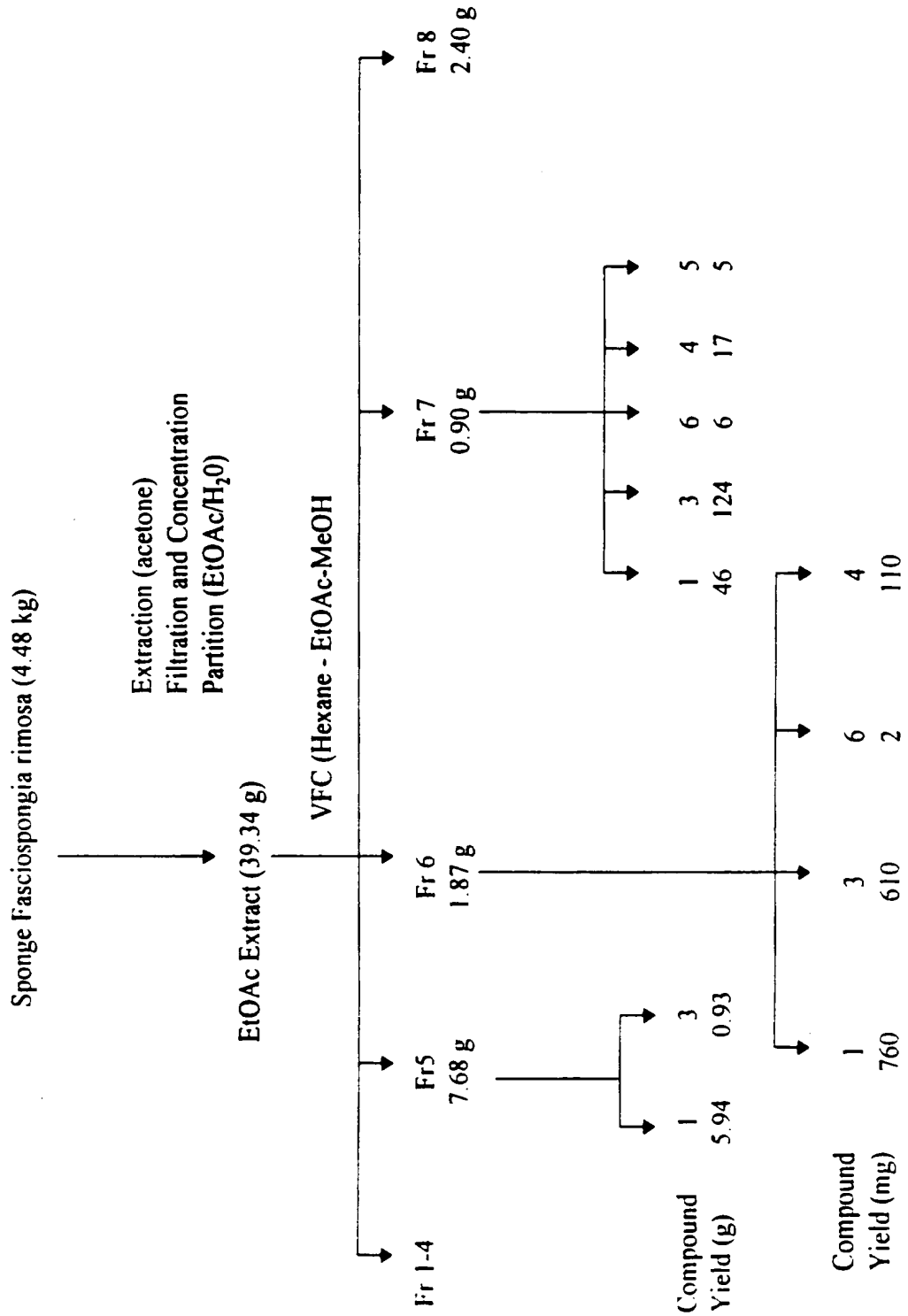
4. A process for preparing a compound selected from the group consisting of latrunculin S, neolaulimalide and zampanolide, also respectively referred to as compounds (5), (6) and (7), of the following formulae:



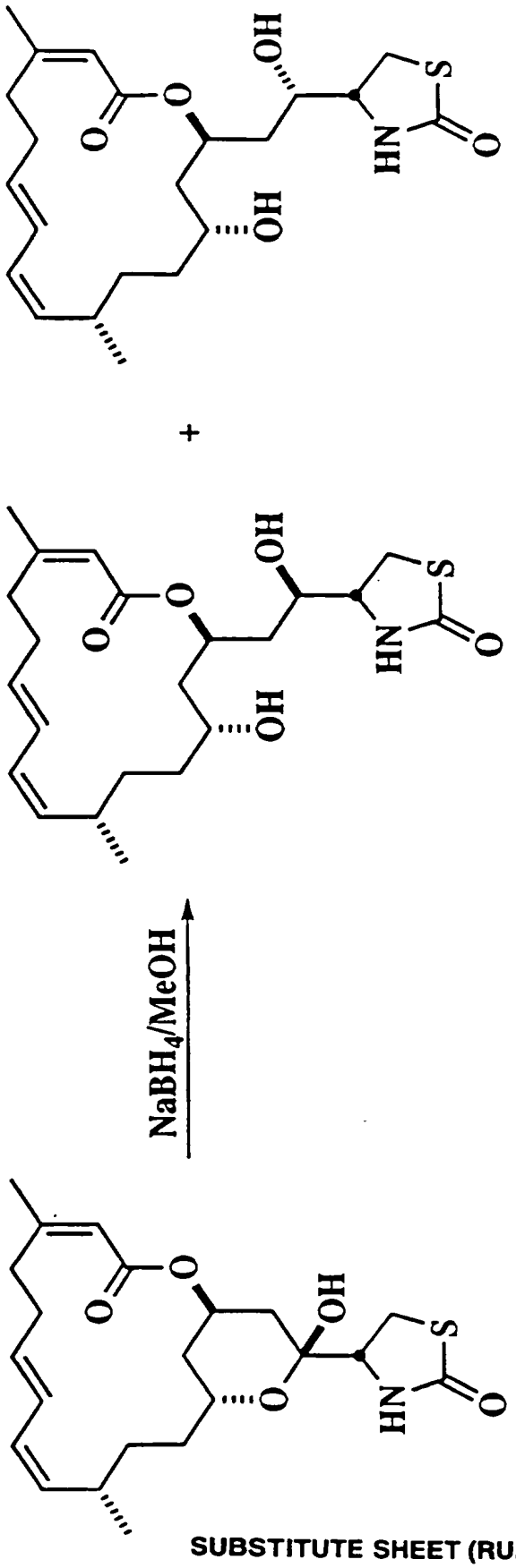


zamapanolide, (7)

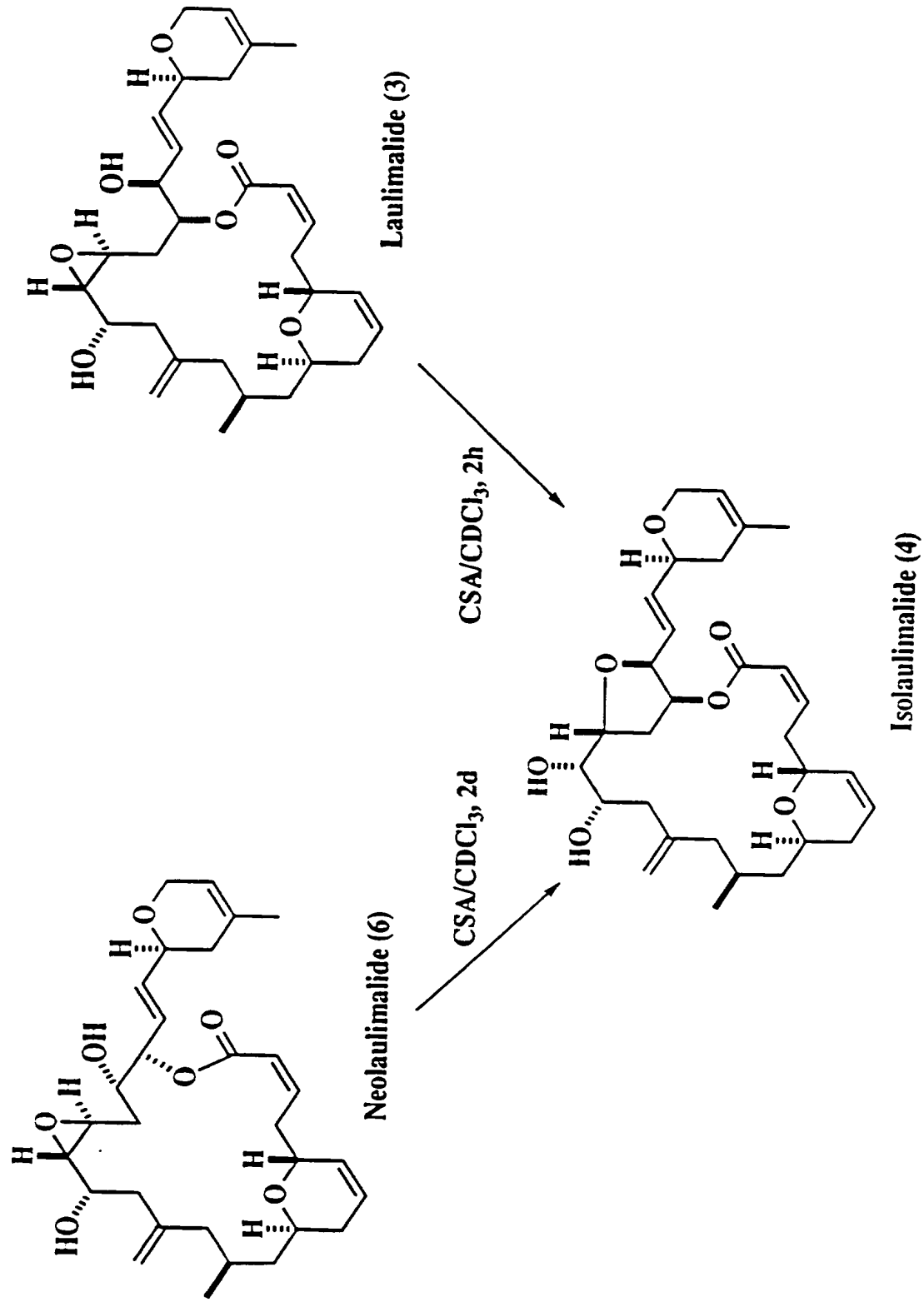
which process comprises isolating the compound from the sponge *Fasciospongia rimosa*.



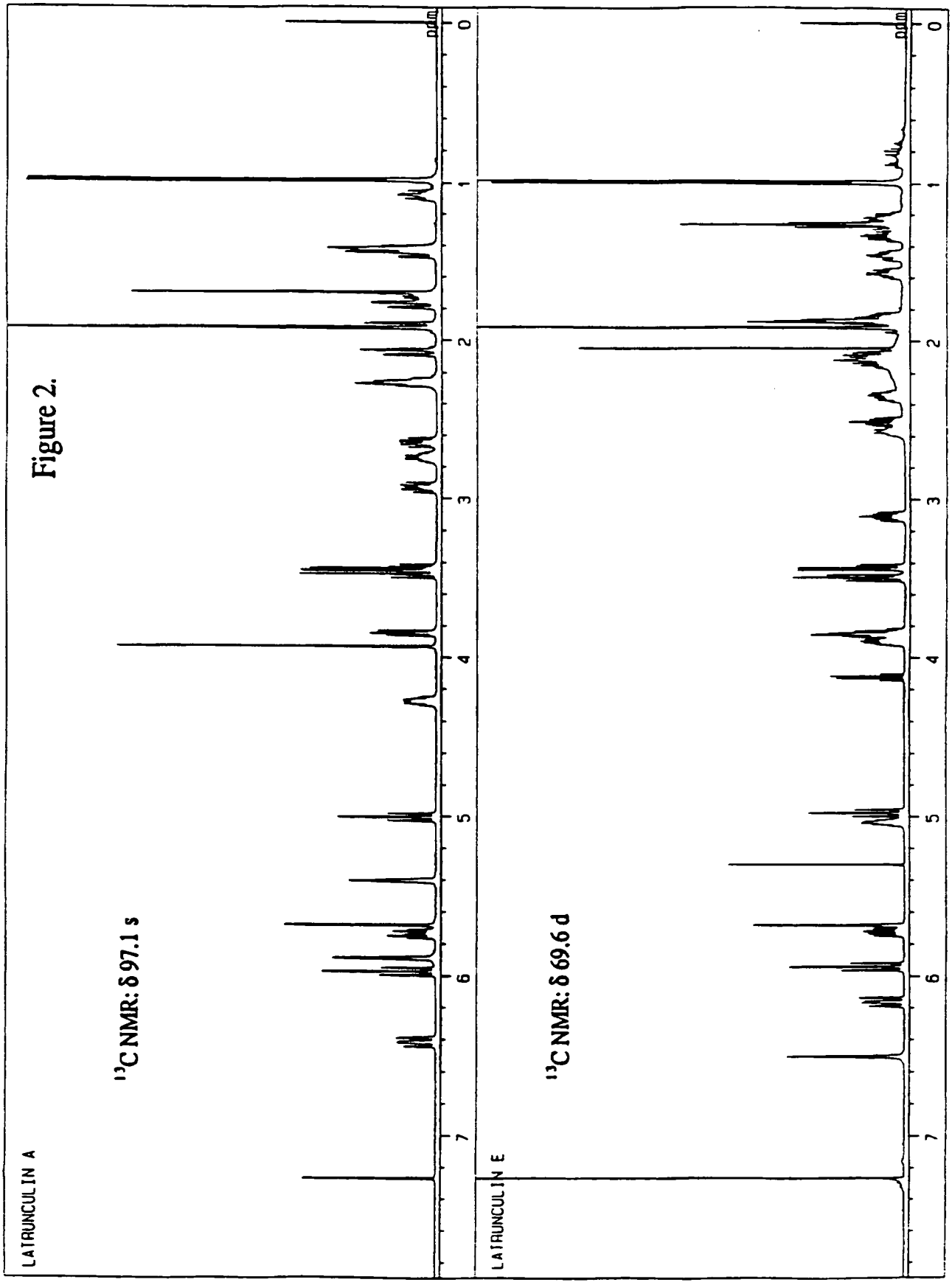
Scheme 1.



Scheme 2.



Scheme 3.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LATRUNCULIN E
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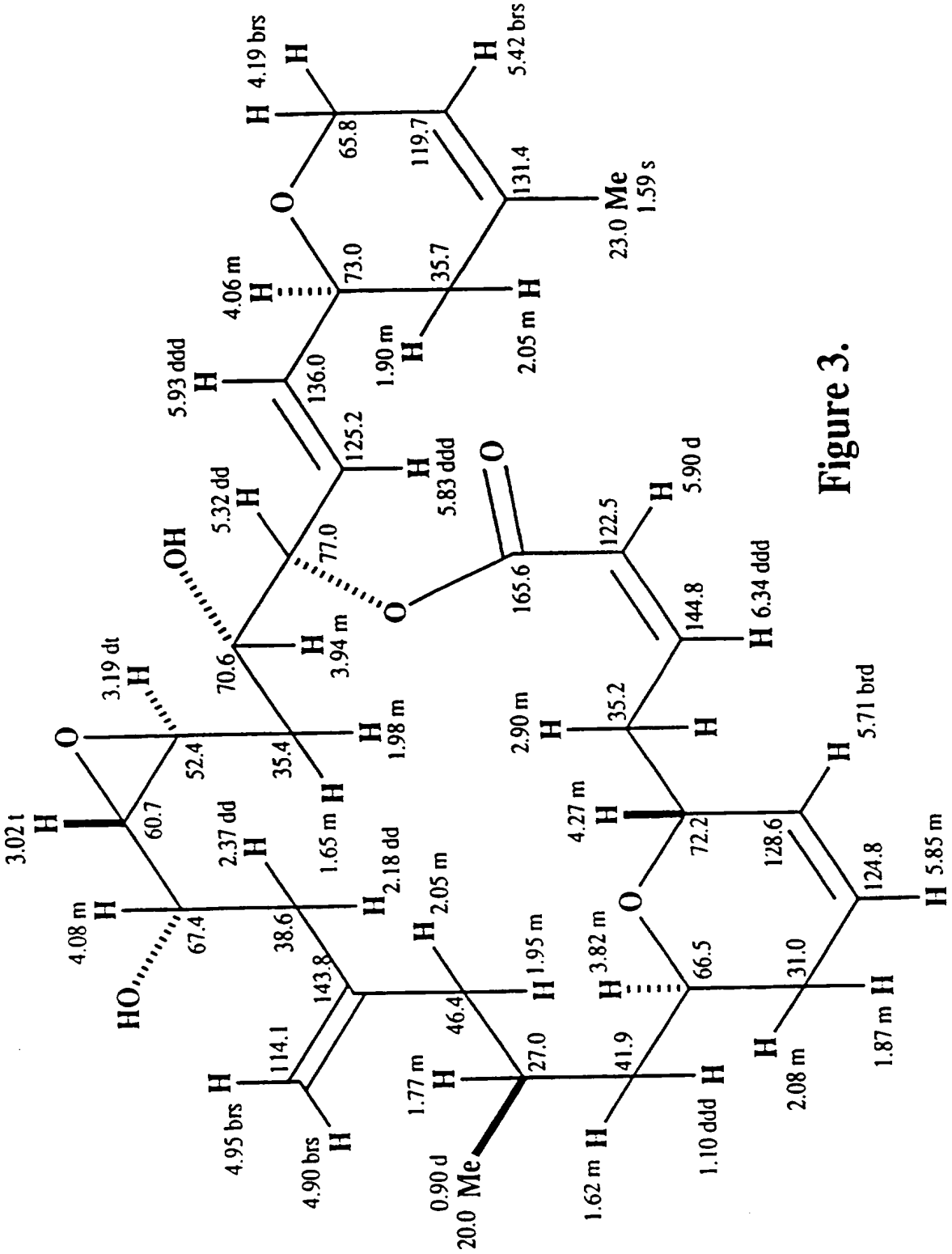
2
3
4
5
6
7

Figure 2.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136.0
1.90 m
2.05 m
125.2
5.32 dd
5.83 ddd
77.0
165.6
122.5
5.90 d
144.8
6.34 ddd
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35.2
3.02 t
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128.6
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4.08 m
67.4
38.6
143.8
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3.82 m
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124.8
5.85 m
114.1
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27.0
41.9
1.77 m
4.95 brs
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0.90 d
20.0 Me
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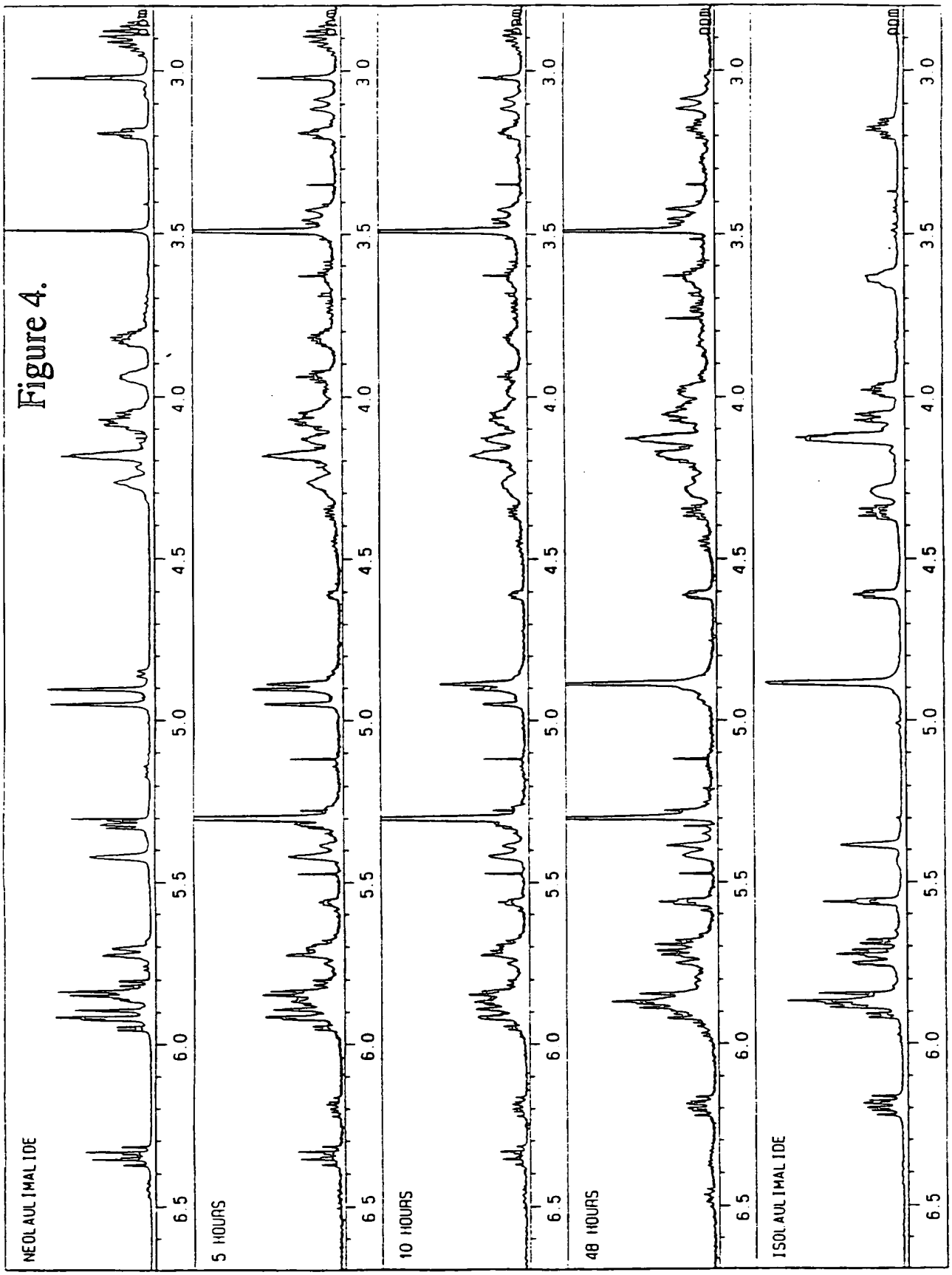
1.10 ddd
2.08 m
1.87 m

Figure 3.

Figure 4.


NEOLAULIMALIDE
5 HOURS
10 HOURS
48 HOURS
ISOLAULIMALIDE
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6.0
5.5
5.0
4.5
4.0
3.5
3.0
ppm

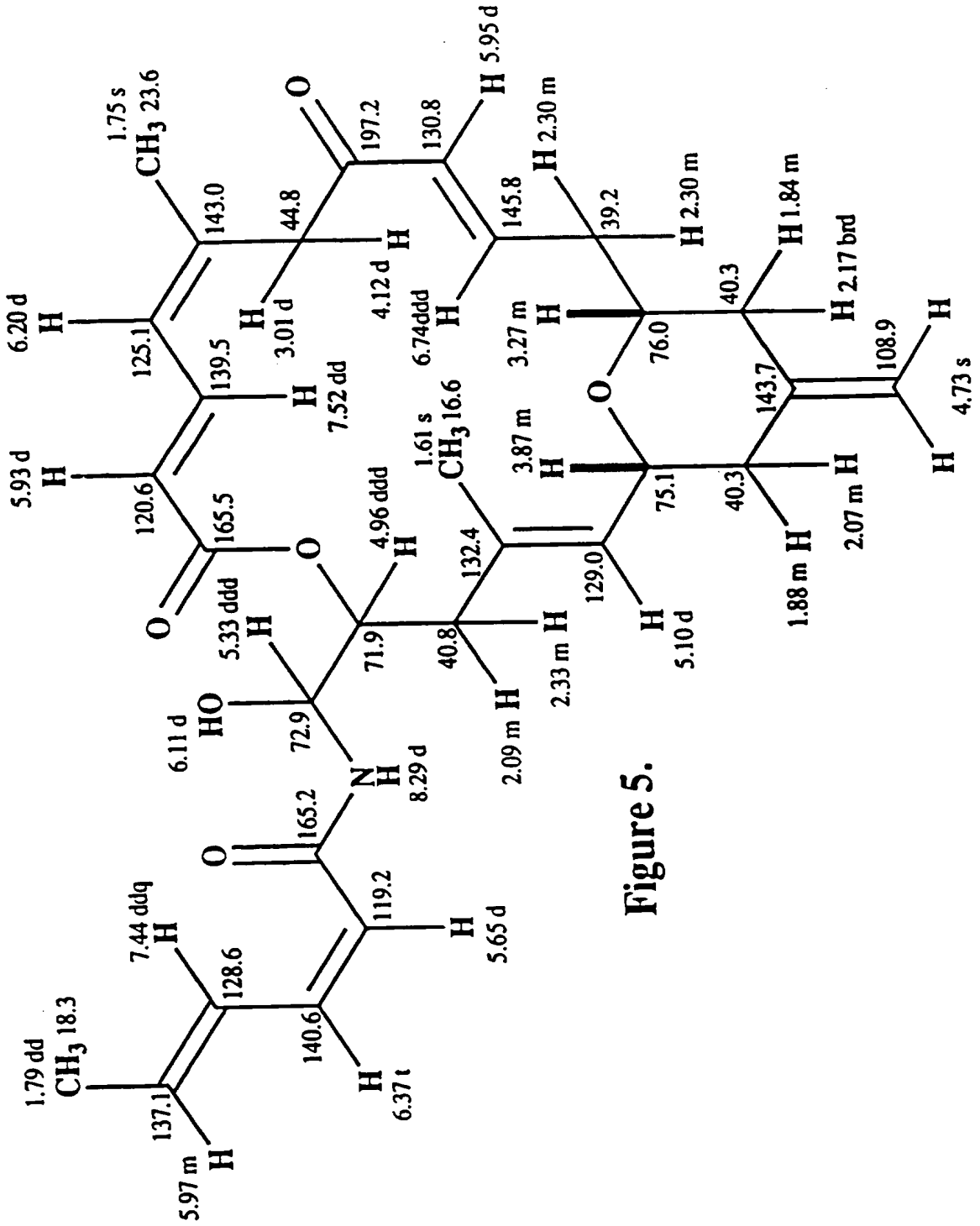

Figure 5.

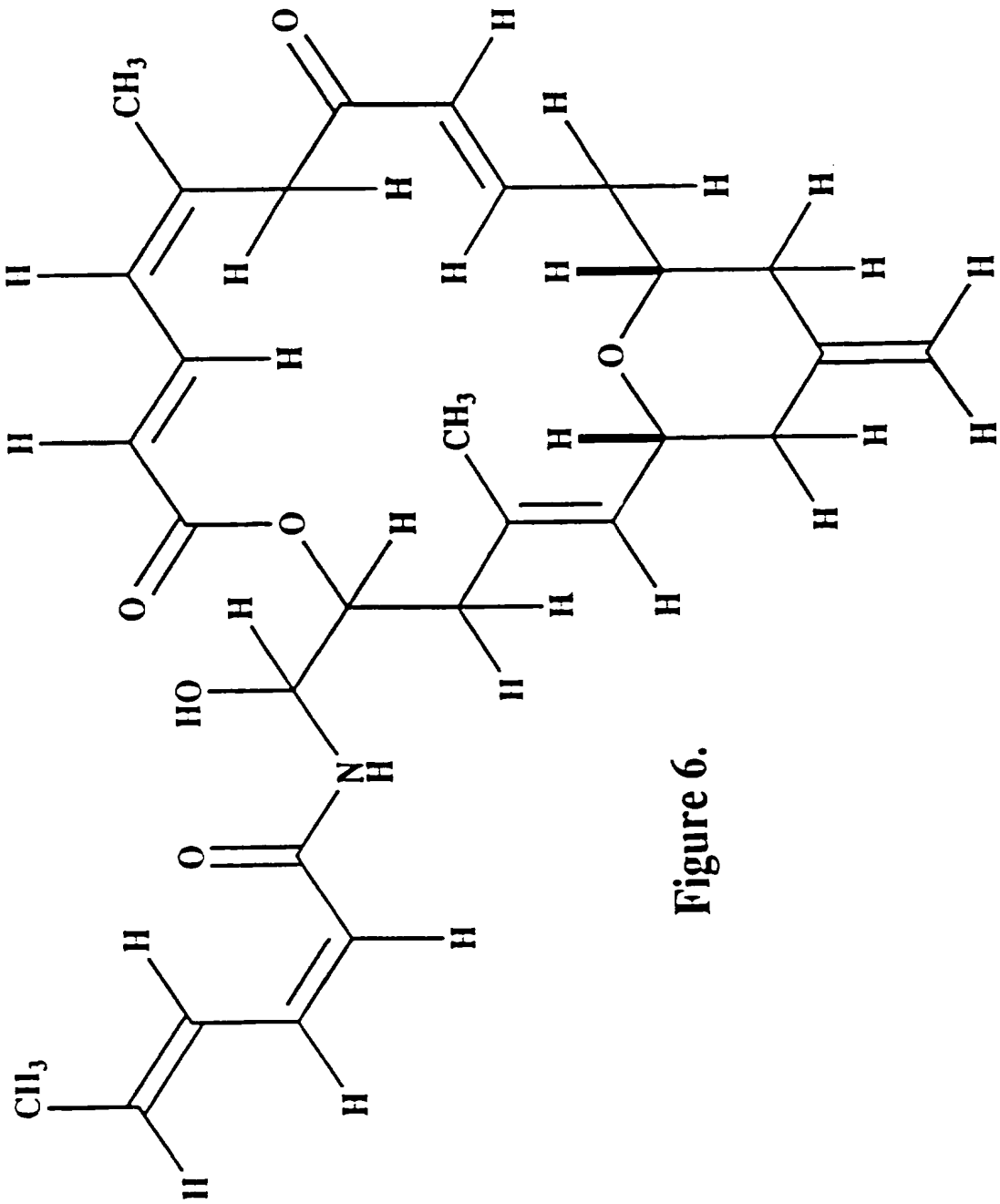

Figure 6.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/02240

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07D417/06 A61K31/335 C07D493/18 C07D493/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. ORG. CHEM., vol. 53, no. 15, 1988, pages 3644-3646, XP002020710 D.G. CORLEY ET AL: "Laulimalides: new potent cytotoxic macrolides ..." cited in the application * complete document * ---	1
A	J. ORG. CHEM., vol. 48, no. 20, 1983, pages 3512-3516, XP002020711 AMIRAM GROWEISS ET AL: "Marine toxins of ..." cited in the application * complete document * --- -/--	1

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1996	18.12.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Van Bijlen, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

national application No.
PCT/GB 96/02240

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 3 is directed to a method of treatment of (diagnosti method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

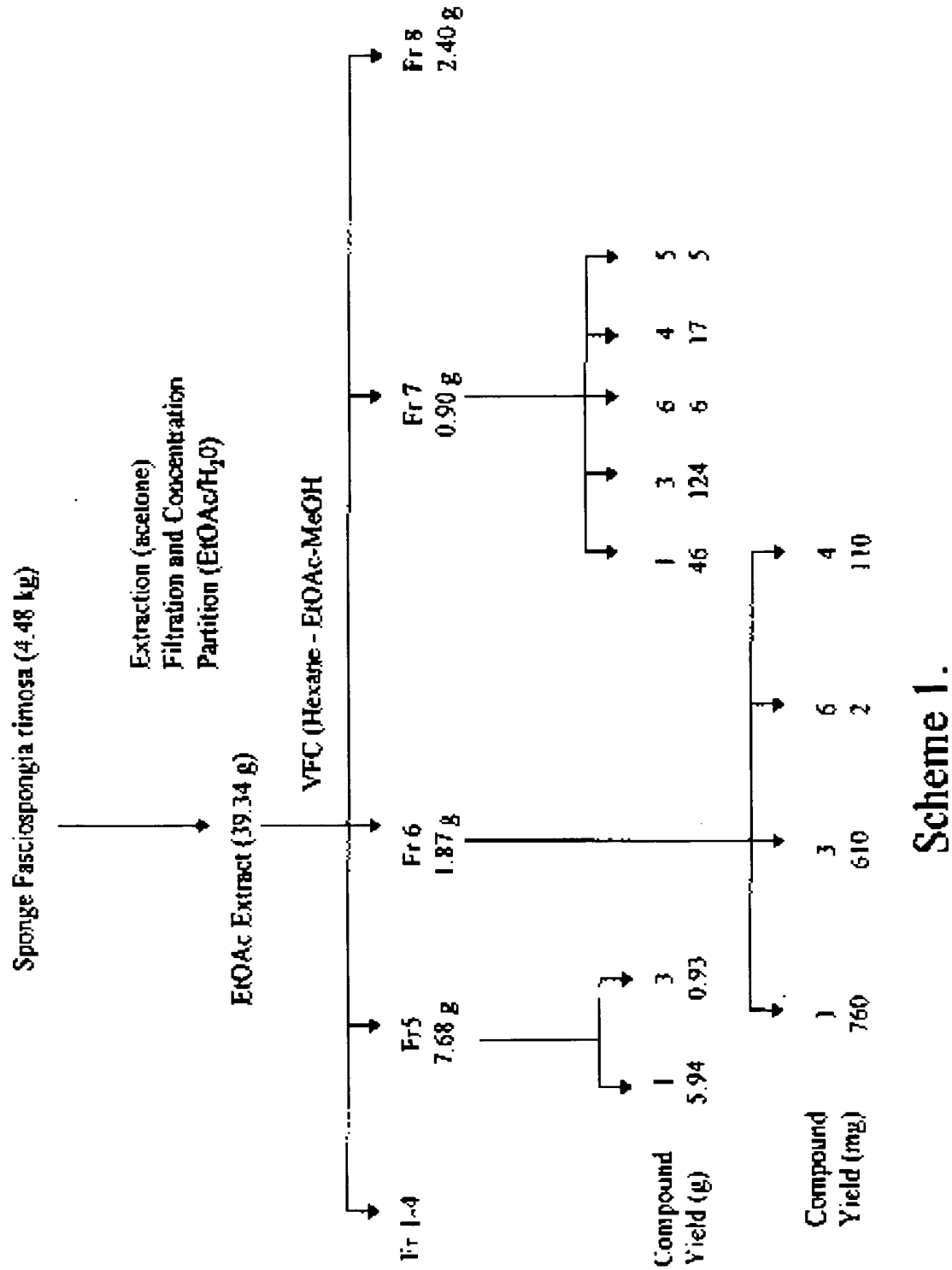
INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/02240

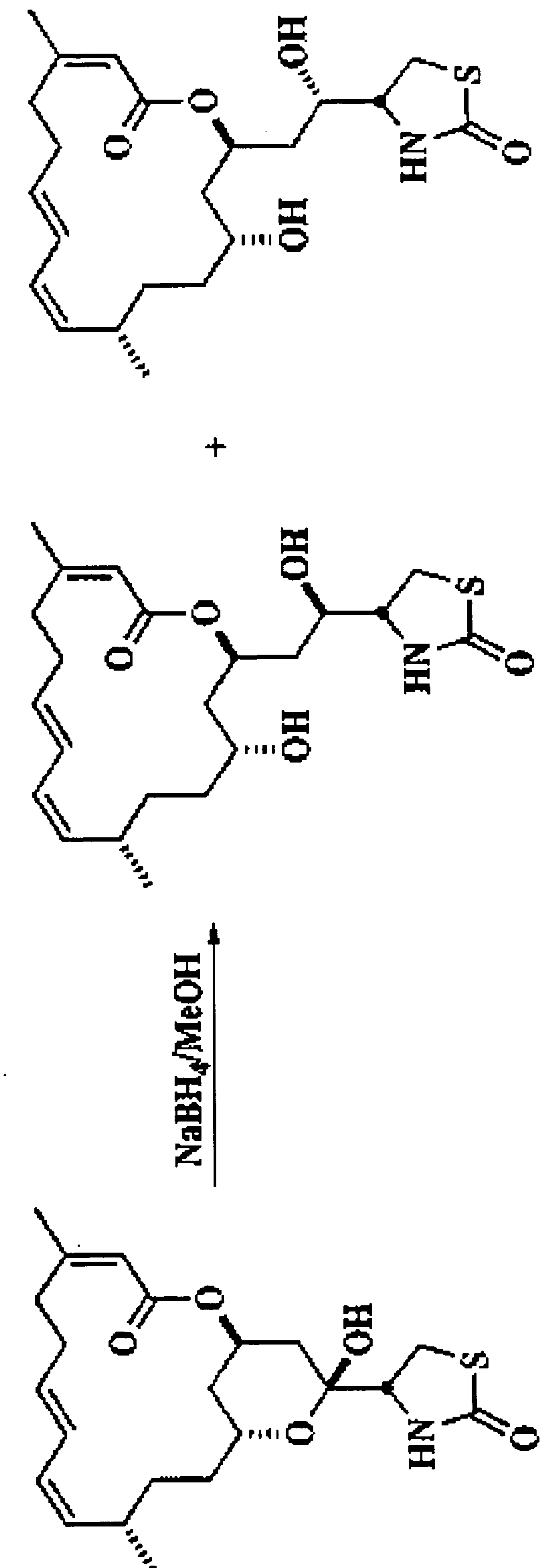
C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. ORG. CHEM., vol. 53, no. 15, 1988, pages 3642-3644, XP002020712 E. QUINOA ET AL: "Fijianolides, polyketide heterocycles from a marine sponge" cited in the application * complete document * ---	1
P,X	CHEMICAL ABSTRACTS, vol. 124, no. 23, 3 June 1996 Columbus, Ohio, US; abstract no. 312574n, TANAKA, JUNG-ICHI ET AL: "New cytotoxic macrolides from the sponge Fasciospongia rimosa" XP002020713 see abstract & CHEM. LETT., vol. 4, pages 255-256, -----	1,2,4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)



Scheme 1.

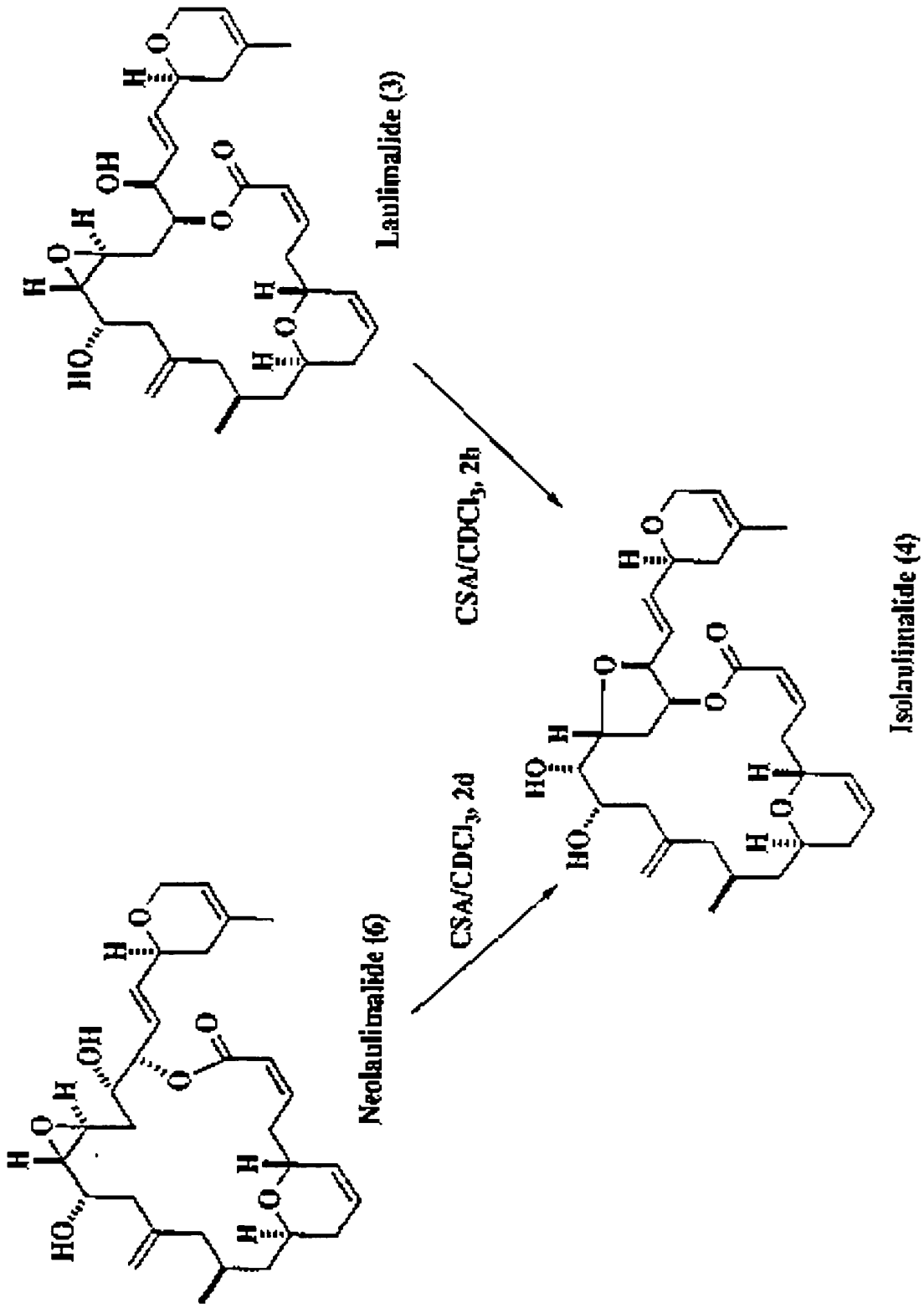


Latrunculin A (1)

Latrunculin S(5)
52 %

17-*Epi*-latrunculin S(8)
42 %

Scheme 2.



Scheme 3.

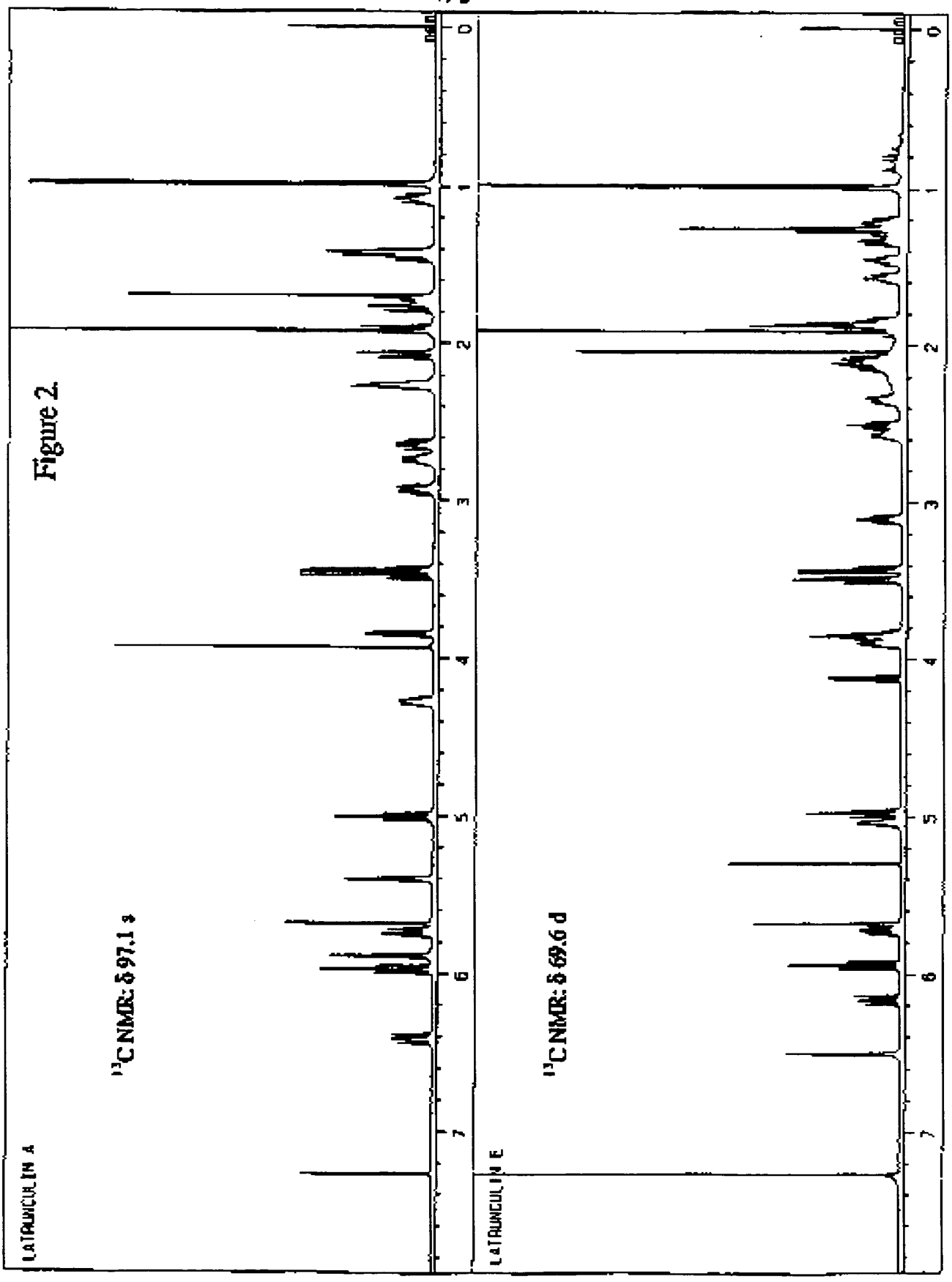

LATRUNCULIN A
^{13}C NMR: δ 97.1 s
LATRUNCULIN B
^{13}C NMR: δ 69.6 d
0
1
2
3
4
5
6
7

Figure 2.

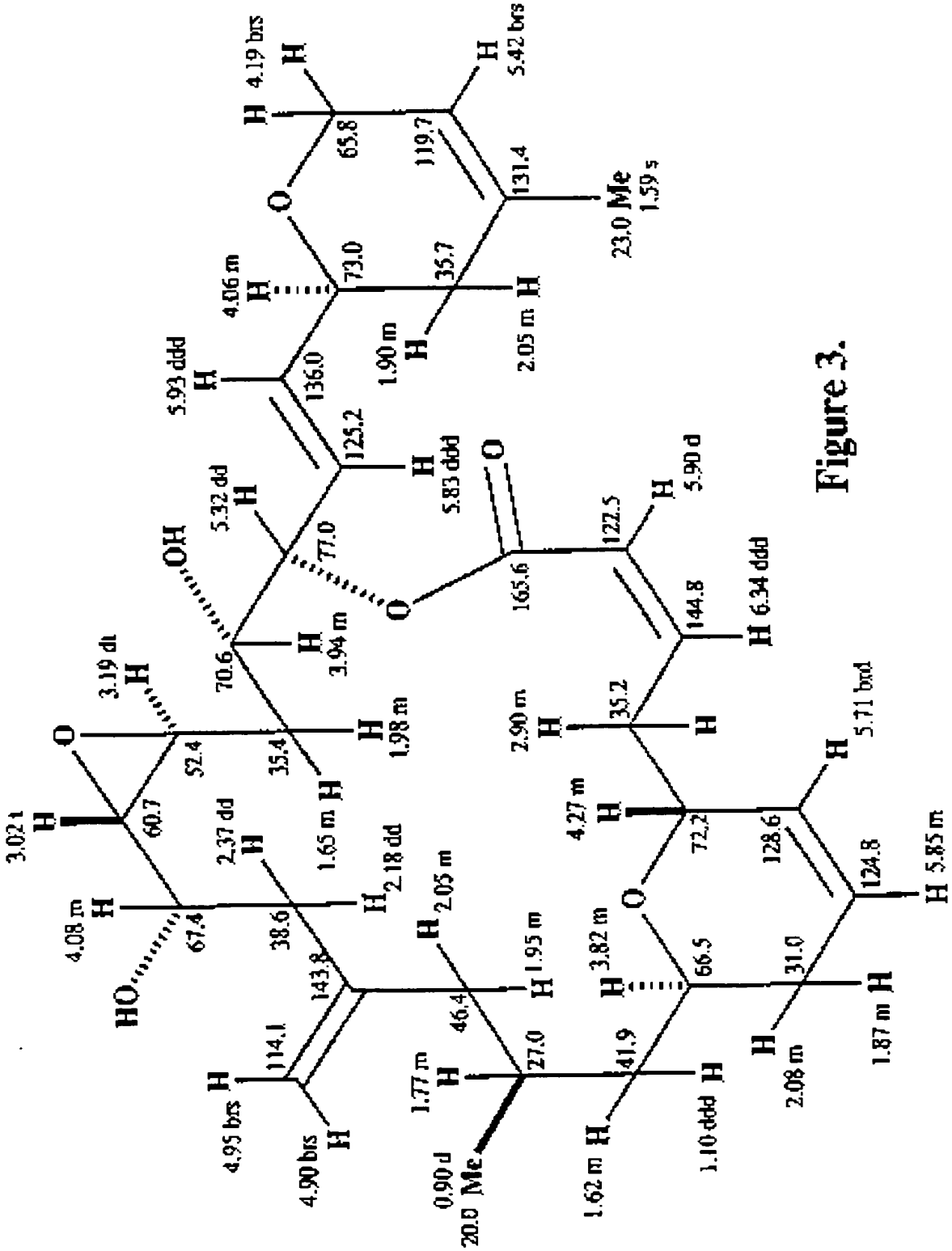
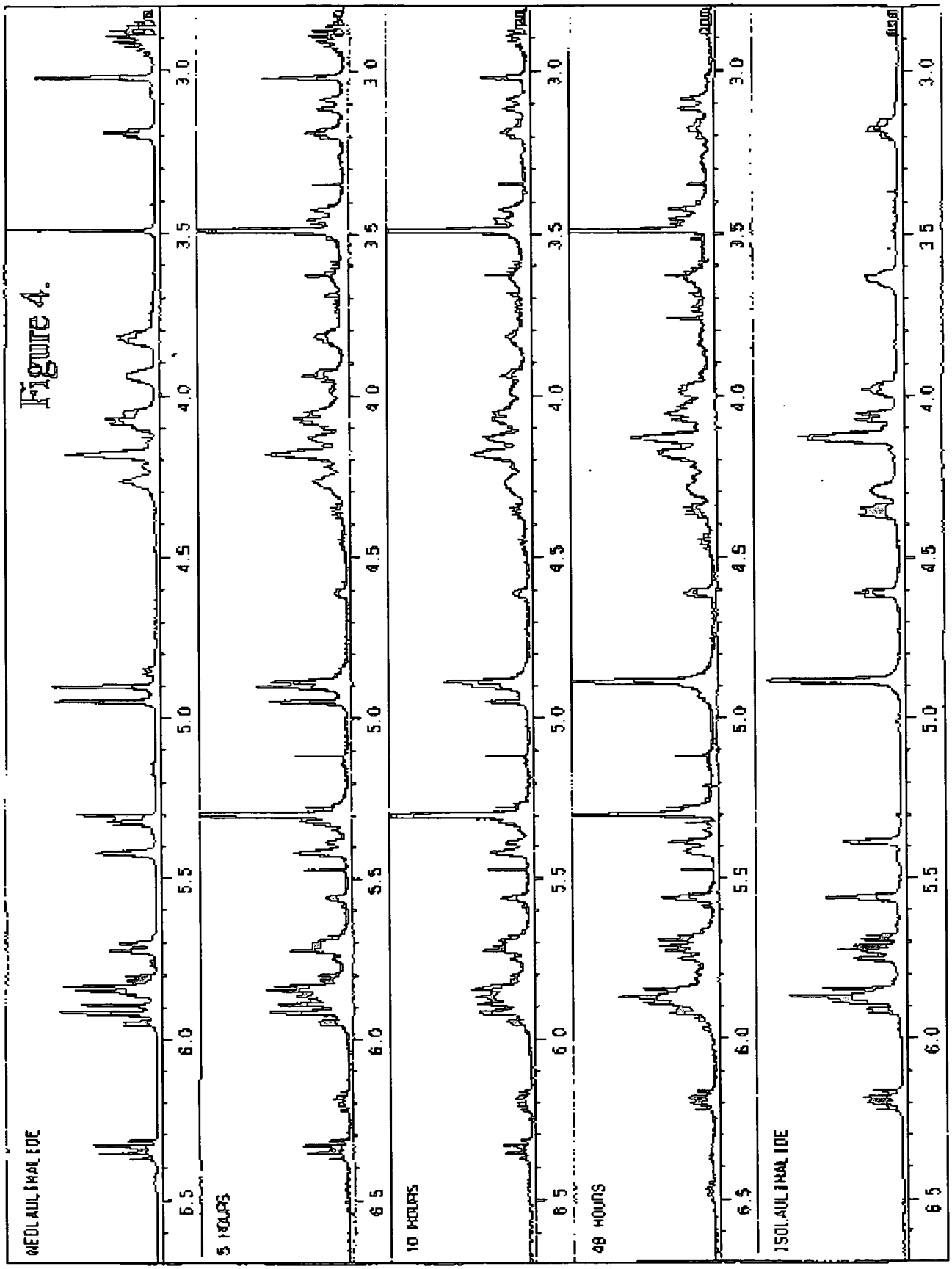

4.19 brs
5.42 brs
65.8
119.7
131.4
23.0 Me
1.59 s
4.06 m
73.0
35.7
5.93 ddd
136.0
1.90 m
2.05 m
125.2
5.83 ddd
5.32 dd
77.0
165.6
122.5
5.90 d
3.94 m
144.8
6.34 ddd
3.19 dt
70.6
2.90 m
35.2
1.98 m
52.4
35.4
5.71 bnd
3.02 t
60.7
4.27 m
72.2
128.6
124.8
5.85 m
2.37 dd
1.65 m
2.18 dd
4.08 m
67.4
38.6
2.05 m
3.82 m
66.5
31.0
143.8
1.95 m
46.4
114.1
1.77 m
27.0
41.9
4.95 brs
4.90 brs
0.90 d
20.0 Me
1.62 m
1.10 ddd
2.08 m
1.87 m
OH
HO

Figure 3.

Figure 4.


MEDIAUL BMAL IDE
5 HOURS
10 HOURS
48 HOURS
ISOLAUL BMAL IDE
5.5
6.0
6.5
4.0
4.5
5.0
3.0
3.5

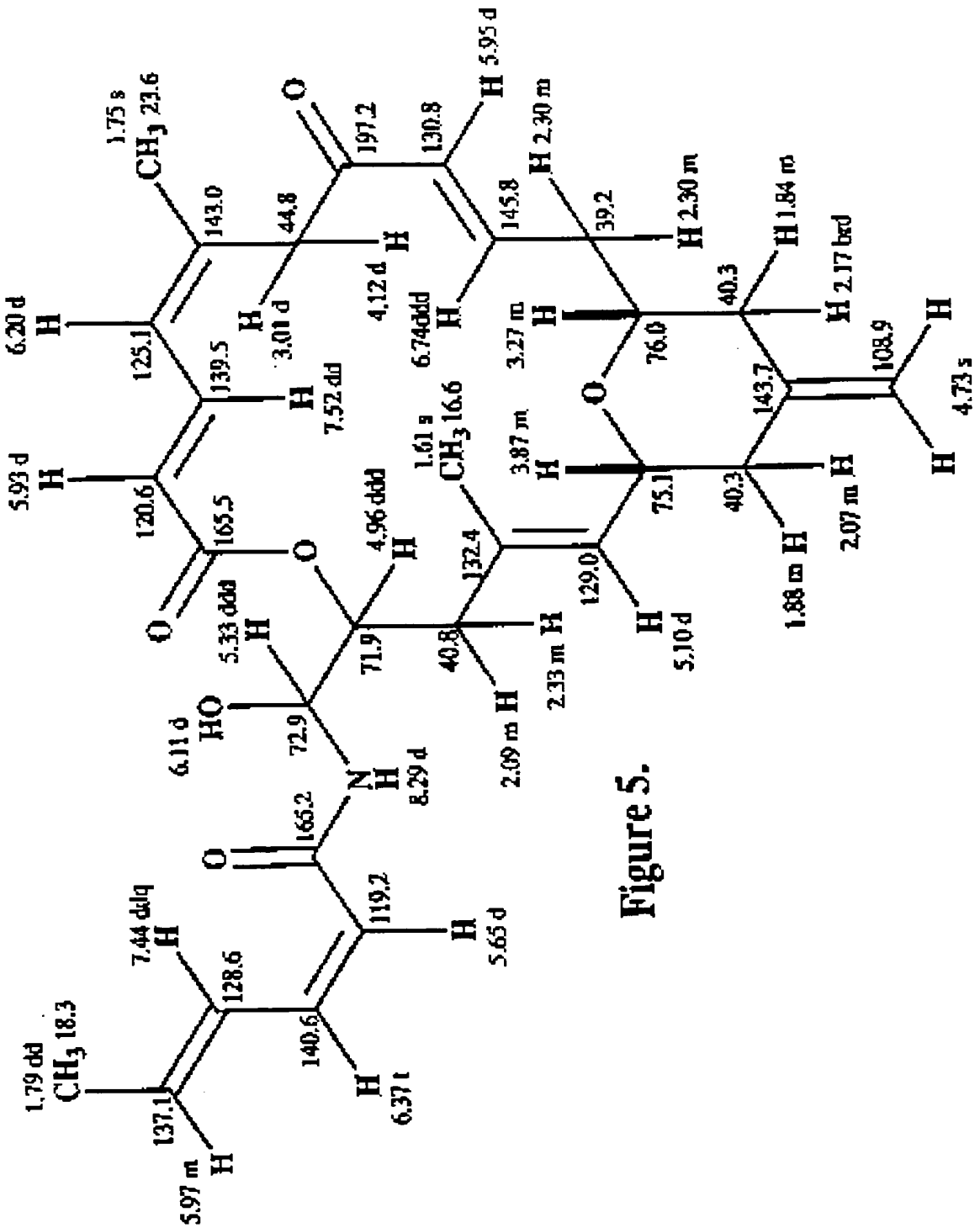


Figure 5.

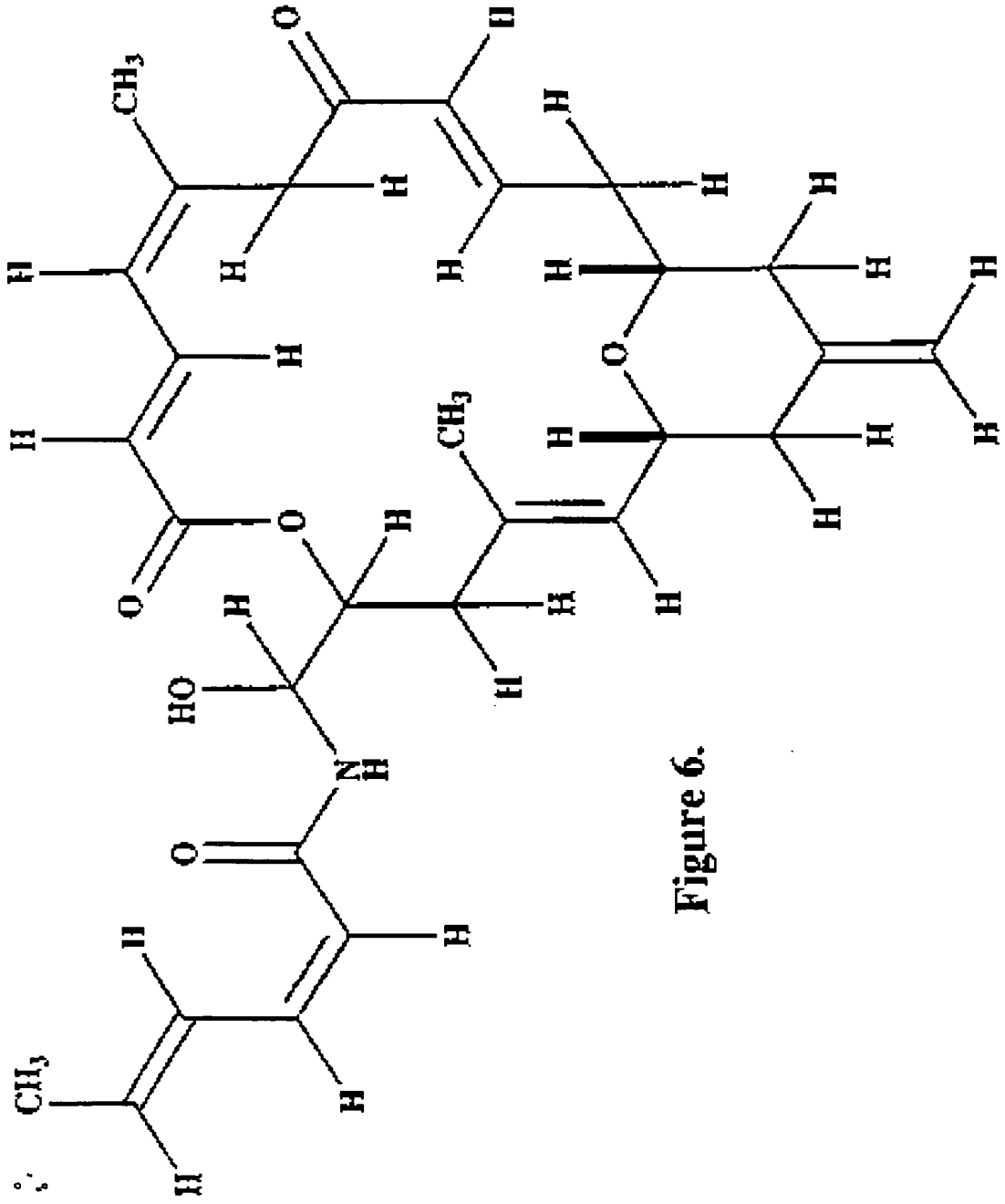


Figure 6.